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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

TITLE: cDNA CLONE FOR TAXADIENE 5-ALPHA-HYDROXYLASE AND METHODS OF USE

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☒ 23 pages of specification are enclosed.

☒ 6 sheet(s) of drawings are enclosed.

☒ Small entity status is claimed for this application.

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cDNA CLONE FOR TAXADIENE 5-ALPHA-HYDROXYLASE AND METHODS OF USE

Summary

The first oxygenation step in the biosynthesis of the anticancer drug taxol in yew (Taxus) species is the cytochrome P450-mediated hydroxylation (with double bond migration) of the diterpene olefin precursor taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5α-ol. A homology-based cloning strategy, employing an induced Taxus cell library, yielded a cDNA encoding taxadiene 5α-hydroxylase which was functionally expressed in yeast and insect cells. The recombinant enzyme was characterized and shown to efficiently utilize both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene (as an adventitious substrate) in the synthesis of taxa-4(20),11(12)-dien-5α-ol. This hydroxylase resembles, in sequence and properties, other cytochrome P450 oxygenases of taxol biosynthesis. The utilization as substrate of both taxadiene isomers in the formation of taxa-4(20),11(12)-dien-5α-ol is novel and suggests a reaction mechanism involving promiscuous radical abstraction with selective oxygen insertion, rather than epoxidation of the C4,C5-double bond of the natural substrate and allylic rearrangement of the resulting taxa-11(12)-en-4,5-epoxide.

Running Title

Cytochrome P450 taxadiene 5α-hydroxylase

Keywords

Biosynthesis; Taxadiene hydroxylase; Regioselective hydrogen radical abstraction; Taxa-4(5),11(12)-diene; Taxa-4(20),11(12)-diene; Taxa-4(5),11(12)-dien-5α-ol; Paclitaxel; Recombinant cytochrome P450 oxygenase; Taxus; Yew

Introduction

Taxol [1] (generic name paclitaxel, **1**, Figure 1) is well-established as a potent chemotherapeutic agent with excellent activity against a range of cancers [2, 3]. This diterpenoid, derived from

yew (Taxus) species [4], continues to find wide application, both in treatment of additional cancer types and for earlier disease intervention [2, 3, 5]. Total syntheses of Taxol have been achieved by several elegant routes (see [6] for recent review) but the yields are too low to be practical, and it has been clear for some time [7, 8] that the supply of Taxol and its semisynthetically useful precursors [6] must continue to rely on biological methods of production for the foreseeable future. Improvement of the biosynthetic process in intact yew or derived cell cultures [9-11] should be based upon a full understanding of the pathway for Taxol formation, the enzymes which catalyze this extended sequence of reactions and their mechanisms of action, and the structural genes encoding these enzymes, especially those responsible for slow steps of the pathway.

Biochemical studies with cell-free enzyme extracts of yew have demonstrated that the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (2) to the committed Taxol precursor taxa-4(5),11(12)-diene (3) [12, 13] is followed by cytochrome P450-mediated hydroxylation at C5 of the olefin with double bond rearrangement [14] to yield taxa-4(20),11(12)-dien-5α-ol (4) (Figure 1). This unusual hydroxylation reaction at C5 is the first of eight cytochrome P450-catalyzed oxygenations and four CoA-dependent acylations that decorate the taxadiene core en route to Taxol (1) [15].

A range of reverse genetic and homology-based cloning strategies [16, 17] has been employed to isolate cDNAs encoding taxadiene synthase [18], from which the recombinant enzyme [19] was utilized to define the mechanism of this novel cyclization [20, 21], and a number of regiospecific acyltransferases of the Taxol pathway [16, 17, 22]. In the case of the cytochrome P450 oxygenases, an alternative to difficult reverse genetic cloning was taken based on differential display of mRNA-reverse transcription-PCR using cultured Taxus cells induced for Taxol production as the source [23], and with sorting of clones by sequence relatedness and functional expression [24]. This strategy yielded a family of 16 oxygenases from which cDNAs encoding the cytochrome P450 taxoid 10β-hydroxylase [24], 13α-hydroxylase [25], and a side-route taxoid 14β-hydroxylase [26] were obtained. Detailed evaluation of this set of heterologously expressed clones failed to yield the taxadiene 5α-hydroxylase. However, because the 5α-hydroxylase is only weakly induced (<two-fold) in methyl jasmonate-treated cell cultures [15], it is possible that the corresponding message was missed by the differential display-based cloning technique [24].

As an alternative for acquiring the taxadiene 5α-hydroxylase gene, a homology-based cloning approach was utilized in which screening probes were generated by PCR using primers directed to regions of very high sequence conservation in cytochrome P450 oxygenases of plant origin. This generic strategy provided suitable probes for screening the induced Taxus cell cDNA library, from which the most abundant of the three new clones obtained was expressed in yeast and shown to encode the target 5α-hydroxylase. This cytochrome P450 hydroxylase, which catalyzes the initial oxygenation step of Taxol biosynthesis and resembles other taxoid hydroxylases, was shown to employ with good efficiency both taxa-4(5),11(12)-diene (with allylic rearrangement) and taxa-4(20),11(12)-diene (directly) in the conversion to taxa-4(20),11(12)-dien-5α-ol. The utilization of the 4(20),11(12)-olefin isomer by this enzyme, although it is not a physiologically relevant substrate in Taxus, allowed mechanistic definition of this unusual cytochrome P450-catalyzed reaction.

Results and Discussion

Homology-Based Cloning of Cytochrome P450 Oxygenases from Taxus

To obtain cytochrome P450 taxoid oxygenase clones that may have been missed by the differential display method [24] due to their low level of induction [15], a general cloning strategy directed to this gene type was employed based upon the highly conserved PERF motif and the region surrounding the heme-binding, invariant cysteine residue of these enzymes [27-29]. PCR amplification, using degenerate primers designed to these amino acid sequences and the previously described Taxus cell cDNA library as template, yielded amplicons of the expected length (~170 bp) which were cloned and sequenced, and shown to resemble other cytochrome P450s. Based on these partial sequences, labeled oligonucleotide probes were synthesized and used to screen the aforementioned Taxus cell cDNA library. Over 30 positive plaques were purified by 3 rounds of hybridization, and following excision, cloning and sequencing led to the identification of several of the cytochrome P450 sequences previously obtained by differential display [24]. However, three new, full-length sequences were also acquired, one of which (designated S1) represented the most abundant cytochrome P450 cDNA isolated by this generic homology-based approach.

The S1 clone of 1688 bp (GenBank accession no. AY289209) contained an apparent ORF of 1509 bp encoding a predicted protein of 502 aa with deduced molecular weight of

56,859, subsequently shown (see below) to be in good agreement with the size of the expressed enzyme observed by SDS-PAGE (~57 kDa). The deduced amino acid sequence of clone S1 exhibited characteristics typical of cytochrome P450 enzymes [30], including the oxygen-binding domain, an N-terminal membrane anchor, the highly conserved heme-binding motif with PFG element (aa437-aa439), and the absolutely conserved cysteine at position 445. Deduced sequence comparisons with previously defined cytochrome P450 taxoid hydroxylases, including the taxoid 10β-hydroxylase (AF318211), 13α-hydroxylase (AY056019) and 14β-hydroxylase (AY188177), revealed overall identities in the 61-63% range and similarities in the 79-81% range (Figure 2), suggesting that clone S1 encoded a new taxoid oxygenase.

Functional Expression of Cytochrome P450 Clone S1 in Yeast

For test of function of the encoded enzyme, we exploited the simplicity and reliability of heterologous expression of cDNA clone S1 in Saccharomyces cerevisiae strain WAT11 which harbors a galactose-inducible NADPH-cytochrome P450 reductase from Arabidopsis thaliana that is required for efficient reductive coupling to the cytochrome [31]. This system also permits test of catalytic activity by in vivo feeding of taxoid substrates to the transformed yeast [24], thereby eliminating the need for microsome isolation in the preliminary functional screen. For this purpose, the ORF of clone S1 was transferred to the pYES2.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) for expression in the yeast host and monitoring via immunoblot analysis of the resulting fusion protein created by joining the S1 ORF (deleted stop codon) to the vector-encoded simian V5 epitope and C-terminal His_6-tag. The suitability of this C-terminal tagging protocol has been demonstrated in the functional expression of other cytochrome P450 taxoid hydroxylases [26].

Transformed yeast cells, confirmed to express the recombinant S1 cytochrome P450 by immunoblot analysis of membrane protein extracts, were tested for catalytic function of the expressed oxygenase by in vivo feeding of exogenous taxane substrates, including taxa-4(20),11(12)-dien-5α-ol, taxa-4(20),11(12)-dien-5α-yl acetate, taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol, taxa-4(20),11(12)-dien-5α,13α-diol, taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol), and both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene [32]. As a negative control for the feeding experiments, the yeast host was transformed with the same vector containing a β-glucuronidase insert instead of the cytochrome P450 S1 clone. Extracts of the overnight reaction mixtures were then analyzed by radio-HPLC,

by which it was shown that only the two taxadiene isomers were efficiently (almost quantitatively in the case of the 4(5),11(12)-isomer) converted to more polar products. In the case of taxa-4(5),11(12)-diene as substrate, the principal biosynthetic product (>92%) eluted with a retention time identical to that of taxa-4(20),11(12)-dien-5α-ol [14, 32] and the minor product (<5%) eluted with a retention time consistent with that of a taxadien-diol. GC-MS analysis (electron impact ionization) confirmed the major product to possess a retention time and mass spectrum identical to that of authentic taxa-4(20),11(12)-dien-5α-ol [14] with characteristic ions at m/z 288 (P^+), 273 (P^+-CH_3), 270 (P^+-H_2O) and 255 (P^+-H_2O-CH_3). The minor, more polar product yielded a mass spectrum consistent with that of a taxadien-diol (ions corresponding to the loss of a methyl and two molecules of water from an unobserved parent ion of m/z 304); however, neither the retention time nor spectrum matched that of available taxadien-diol standards, and too little of this material was available for structural definition by NMR spectrometry.

In the case of taxa-4(20),11(12)-diene as substrate, the major product (~90%) was again shown, upon radio-HPLC analysis, to possess a retention time identical to that of taxa-4(20),11(12)-dien-5α-ol, and this identification was confirmed as before by GC-MS analysis. The unidentified taxadien-diol side product was also observed (~8%), as were a range of other minor metabolites (at ~2% of the product mix) that were also derived from this substrate in the negative control (yeast that expressed β-glucuronidase). These negative controls did not produce taxa-4(20),11(12)-dien-5α-ol or the unidentified taxadien-diol from either taxadiene isomer. To further support the identity of the biosynthetic taxa-4(20),11(12)-dien-5α-ol, this radiolabeled product was isolated by HPLC and the purified material was fed to yeast that functionally express the previously characterized taxoid 13α-hydroxylase [25], whereupon this precursor was quantitatively converted to taxa-4(20),11(12)-dien-5α,13α-diol, as expected. These results confirmed that cytochrome P450 clone S1 encodes a taxadiene 5α-hydroxylase that catalyzes the first oxygenation step of the Taxol biosynthetic pathway.

Characterization of the Recombinant Hydroxylase

To prepare sufficient enzyme for comparative analysis of substrate binding and kinetic phenomena, in a host less prone to interfering activity and artefact formation, the taxadiene 5α-hydroxylase S1 cDNA clone was transferred to the baculovirus-Spodoptera fugiperda (Sf9) expression system (which also coexpresses a Taxus cytochrome P450 reductase) previously

utilized for taxoid hydroxylases [25]. Based on CO-difference spectra [33] of microsomes isolated from Sf9 insect cells expressing the recombinant hydroxylase (versus microsomes from negative control cells expressing the β-glucuronidase gene), in excess of 300 pmol cytochrome P450/mg microsomal protein was routinely produced by this system. Binding spectra [34] for both taxadiene isomers (in the absence of NADPH) were then recorded using these insect cell microsomes enriched in the recombinant hydroxylase. Evaluation of the substrate binding constant (K_s) over a 100-fold range of substrate concentrations showed K_s to vary somewhat from 3 to 5 μM for taxa-4(20),11(12)-diene and from 5 to 8 μM for taxa-4(5),11(12)-diene (a typical data set at 200 pmol protein concentration is illustrated in Figure 3). These results indicate that both positional isomers of the olefin substrate bind with high affinity to the active site of taxadiene 5α-hydroxylase but that taxa-4(20),11(12)-diene is preferred by a factor of about 2.

Kinetic constants for both isomers were next evaluated (at a saturating 200 μM concentration of NADPH plus regenerating system [35]) using the Michaelis-Menten method. Plotting the lines of best fit ($R^2 > 0.99$) provided a K_m value of 16 ± 3.2 μM, with V_{rel} of 120, for taxa-4(20),11(12)-diene, and a K_m value of 24 ± 2.5 μM, with V_{rel} of 100, for taxa-4(5),11(12)-diene (Figure 4); the latter K_m value compares to a K_m value of ~6 μM determined previously for the 4(5),11(12)-isomer with the native enzyme measured in microsome preparations from yew stem tissue [14]. It is notable that the recombinant hydroxylase expressed from the baculovirus-Spodoptera system, and assayed in isolated microsomes, produced only the 5α-hydroxy taxadiene from either olefin substrate, and that the taxadien-diol produced from these substrates in the intact yeast system was not observed. The diol product was thus attributed to the action of yeast host enzyme(s) upon taxadienol; this observation was independently verified by feeding studies with control yeast cells. Comparison of catalytic efficiencies (V_{rel}/K_m) for both substrates with the recombinant enzyme indicated that taxa-4(20),11(12)-diene was preferred to the 4(5),11(12)-isomer by a factor of about 2.

Substrate Utilization by the Native Hydroxylase

Because studies with the recombinant 5α-hydroxylase indicated that both taxadiene isomers were functional substrates in the regiospecific formation of taxa-4(20),11(12)-dien-5α-ol, the native enzyme from induced Taxus cell microsomes was re-evaluated, this system having never been tested previously with the 4(20),11(12)-diene isomer [14]. Microsomes from induced cultured

cells (this starting material is preferred to stem tissue) were isolated as previously described [14] and, following the confirmation of linear reaction conditions in protein concentration and time, kinetic constants were determined for both [20-^{3}H]taxa-4(5),11(12)-diene and [20-^{3}H]taxa-4(20),11(12)-diene with the optimized assay [14]. The radio-HPLC-based assay previously described [15] was employed here to permit summing of taxadien-polyols derived subsequently from the initially formed taxadienol product generated by this microsomal system that contains all of the downstream cytochrome P450 taxoid oxygenases of the pathway [15]. It should be noted that any kinetic isotope effect (KIE) resulting from the C20 deprotonation of [20-^{3}H]taxa-4(5),11(12)-diene was not considered here because previous studies with [20-$^2H_3$]taxa-4(5),11(12)-diene (>99 atom % ^{2}H) indicated that hydrogen removal from C20 is apparently not rate limiting in the overall hydroxylation reaction [14]. By this approach, Michaelis-Menten plotting (R^2 >0.98 for the lines of best fit) indicated a K_m value of 48 μM, and V_{rel} of 100, for taxa-4(5),11(12)-diene, and a K_m value of 27 μM, with V_{rel} 150, for taxa-4(20),11(12)-diene (Figure 4). Thus, in terms of catalytic efficiency (V_{rel}/K_m), the presumed unnatural taxa-4(20),11(12)-diene isomer was again preferred as substrate in the hydroxylation to taxa-4(20),11(12)-dien-5α-ol by this native enzyme, consistent with results using the recombinant form of the enzyme.

Search for a Taxadiene Isomerase and Mechanism of Oxygenase Action

The utilization of taxa-4(20),11(12)-diene as a hydroxylase substrate raised the issue of whether this isomer could be a productive intermediate in vivo, in spite of the fact that the Taxus taxadiene synthase (native and recombinant enzyme) produces principally taxa-4(5),11(12)-diene (94%), with very low level coproduction of taxa-4(20),11(12)-diene (4.8%) and verticillene (1.2%), and only trace amounts of taxa-3(4),11(12)-diene [19]. Two recently acquired taxadiene synthase isoforms (the expressed enzymes of two less abundant allelic variants, Accession No. _____ and Accession No. _____) produce (under established assay conditions [19]) a similar distribution of olefins, thus ruling out this route for the generation of taxa-4(20),11(12)-diene in significant amounts in vivo.

No isomerization of taxa-4(5),11(12)-diene to the 4(20),11(12)-diene isomer (or vice versa) was observed in Taxus cell microsomes (or Spodoptera microsomes enriched in the recombinant 5α-hydroxylase) under standard assay conditions but in the absence of NADPH or in the absence of O_2 (N_2 atmosphere plus and O_2 scavenging system), or in the presence of CO,

100 μM miconazole, or 100 μM clotrimazole (all conditions under which hydroxylation activity is negligible), nor was isomerization observed in boiled controls containing all cofactors and reactants. Similarly, no interconversion of either positional isomer was observed in the presence of magnesium ion, NAD^+, NADH or $NADP^+$, or flavin cofactors, at pH values ranging from 4 to 10. From these studies, we concluded that taxa-4(5),11(12)-diene is not appreciably isomerized to taxa-4(20),11(12)-diene under physiological conditions, and that the migration of the double bond from the 4(5)- to the 4(20)-position in the process of taxadienol formation is an inherent feature of the cytochrome P450 oxygenase reaction with taxa-4(5),11(12)-diene as substrate. It follows that taxa-4(20),11(12)-diene is but a minor natural product of Taxus formed by taxadiene synthase that is an adventitious, yet efficient, substrate for the 5α-hydroxylase.

Previous efforts to evaluate the 5α-hydroxylation reaction by the native enzyme, by search for an epoxide intermediate and through the use of [20-$^2H_3$]taxa-4(5),11(2)-diene to examine a KIE on the deprotonation step, failed to distinguish between the mechanistic possibilities involving preliminary conversion of the 4(5)-double bond of taxa-4(5),11(12)-diene to the corresponding 4(5)-epoxide, followed by ring opening and elimination of a proton from the C20 methyl group to yield the allylic alcohol product, and an alternate route involving cytochrome P450-mediated abstraction of hydrogen from the C20 methyl of the substrate to yield the allylic radical to which oxygen is added at C5 (Figure 5) [14]. The utilization of the isomeric taxa-4(20),11(12)-diene by the hydroxylase, with efficiency comparable to that of the natural substrate, would appear to rule out an intermediate epoxide in the reaction cycle, and would instead suggest a mechanism involving abstraction of a hydrogen radical from C20 (or C5 in the case of the other isomer), leading to the delocalized allylic radical, followed by oxygen insertion selectively from the 5α-face of this radical intermediate to accomplish the rearrangement. Perhaps the somewhat tighter binding of the 4(20)-isomer is a reflection of the ability of this isomer to more closely mimic the allylic radical intermediate.

Significance

Previous attempts to clone the taxadiene 5α-hydroxylase gene, by a method involving differential display of mRNA from induced versus uninduced Taxus cells, failed because, although the corresponding transcript appears to be fairly abundant, the gene is not highly induced and so was missed in the differential screen. A generic homology-based search for

cytochrome P450 cDNA clones yielded the target hydroxylase, thus making available the recombinant enzyme for evaluation of substrate utilization. The efficient conversion of both taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene as substrates allowed us to conclude that the mechanism of this novel oxygenation and rearrangement reaction does not involve initial double bond epoxidation but rather hydrogen abstraction from either of two alternate positions of the olefin isomers to form a common allylic radical intermediate, followed by regio- and stereo-specific oxygen insertion at C5α to yield taxadienol. Based on feeding studies and metabolite analyses, the 5α-hydroxylation is a slow step of Taxol biosynthesis relative to the downstream oxygenations and acylations. Manipulation of the expression of this gene in Taxus should increase pathway flux toward Taxol to improve production yields of this drug from its natural, and currently the only commercially viable, source.

An attractive alternative to yew harvest is the production of Taxol and taxoids in yew cell culture. The practice lends itself to vat fermentation format (potentially as a continuous process), a high level of process control, and ease of product isolation and purification (i.e., it is a "cleaner" system), and it provides the possibility of biochemical/molecular manipulation to direct biosynthesis to specific taxoid precursors, modified forms, and derivatives. In current practice at the small scale, yew cell cultures are capable of producing 10-100 mg/L of Taxol (up to 1 g total taxoids/L) in relatively short production runs (7-10 days), but production levels are quite variable and not sustainable with time or at scale. Commercially viable production levels of Taxol are in the 200-400 mg/L range and of precursors for semi-synthesis in the 400-800 mg/L range. To achieve economic viability, production levels must be enhanced and taxoid metabolism redirected. Production levels must be consistent and reliable, and it is highly desirable that the system be biochemically manipulable so as to permit synthesis of a range of taxoid derivatives (e.g., alternative precursors and second generation drugs). The goal is achieved only by molecular genetic manipulation (genetic engineering) of yew cells to force sustained high level production and to direct the pathway to desirable taxoid metabolites.

To make a yew cell culture-based process a commercial reality, three steps (and the associated experimental tools) are pursued. A reliable gene transformation system and strategy for inserting multiple genes has been developed. A small, critical set of genes form the basis of a transgenic cell culture system for producing Taxol commercially. These genes encode enzymes for unique,

critical steps of the pathway, and include taxadiene synthase (a very slow step in which the taxane skeleton is constructed), taxadiene-5α-hydroxylase (a very slow step for the first oxygenation), taxoid-5α-*O*-acetyl transferase (TAX1, an important step that cannot be done chemically, with the required specificity), and taxoid-2α-*O*-benzoyl transferase (TAX2, an important step that cannot be done chemically, with the required specificity). These genes are used in combination to build a viable production platform by overexpression in cell culture to increase pathway flux towards taxol and increase production yields of this drug in cell cultures.

Experimental Procedures

Enzymes, Substrates and Reagents

Enzymes and reagents were obtained from Gibco/BRL (Grand Island, NY), Invitrogen (Carlsbad, CA), New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA), as indicated, and were used according to the manufacturers' instructions. Other chemicals described were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO). The preparations of (±)-[20-^{3}H]taxa-4(5),11(12)-diene (5.3 Ci/mol), (±)-[20-^{3}H]taxa-4(20),11(12)-diene (2.6 Ci/mol) and (±)-taxa-4(20),11(12)-dien-5α-ol (2.0 Ci/mol) have been described [32], as have the preparations of (±)-taxa-4(20),11(12)-dien-5α-yl acetate (2.0 Ci/mol) [15, 36], (±)-taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol (2.0 Ci/mol) and (±)-taxa-4(20),11(12)-dien-5α,13α-diol (2.0 Ci/mol) [25, 26]; the preparation of (+)-[^{3}H-acetyl]taxusin (the tetraacetate of taxa-4(20),11(12)-dien-5α,9α,10β,13α-tetraol, at 10.0 Ci/mol) will be described elsewhere (M. Chau and R. Croteau, in preparation).

Homology-Based Cloning of Cytochrome P450 Oxygenases

A generic cloning strategy for cytochrome P450 genes [27-29] was employed based upon two highly conserved regions of these proteins, the commonly occurring PERF sequence and the region surrounding the invariant heme-binding cysteine residue [30]. Thus, amino acid sequence alignments of cytochrome P450s of plant origin, available in the public databases, allowed synthesis of degenerate and inosine-containing oligonucleotide primers directed to the PERF motif and its variant forms [i.e., 5'-TTY MGI CCI AGM GIT TYG AR-3' (forward), 5'-TTY MGI CCI TCI MGI TTY GAR-3' (forward), 5'-CKI III CCI GCI CCR AAI GG-3' (forward), 5'-GAR GAR TTY MGN CCN GAR MG-3' (forward), and 5'-GAR AAR TTY III CCI GAI ARG

TTY (forward)] and to the conserved heme-binding region [i.e., 5'-GGR CAI III CKI III CCI CCI CCR AAI GG-3' (reverse), and 5'-CCI GGR CAI ATI MKY YTI CCI GCI CCR AAI GG-3' (reverse)]. Amplification [29], using first strand cDNA template derived from mRNA isolated from T. cuspidata cells 16 h post-induction with methyl jasmonate [23, 24], yielded the predicted amplicons of about 200 bp, and these were gel purified, ligated into pGEM-T (Promega, Madison, WI), and transformed into E. coli JM109 cells for plasmid preparation and insert sequencing. Based on these sequences, 40 to 50 nt long probes were synthesized, 5'-labeled with [^{32}P]dCTP (ICN, Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and used to screen the previously described induced T. cuspidata λ-ZAPII cDNA library [24] by employing Rapid-Hyb (Amersham Pharmacia, Piscataway, NJ) solution. Following 3 rounds of screening, the 32 positives were in vivo excised as pBluescript II SK(–) phagemids by the Stratagene protocol, and partially sequenced using T3 and T7 promoter primers to sort clones into new acquisitions that were not obtained in the prior differential display screen [24]. These new clones were obtained in full-length form [by Marathom 5'-RACE (Clontech, Palo Alto, CA) as necessary], and were fully sequenced.

Cytochrome P450 cDNA Expression in Yeast

For functional expression in Saccharomyces cerevisiae, the deduced ORFs of the cytochrome P450 cDNAs were amplified by PCR using a gene-specific forward primer (containing the ATG start codon) and a corresponding reverse primer in which the stop codon was deleted to permit read-through when transferred to pYES2.1/V5-HIS-TOPO (Invitrogen). By this means, a fusion protein was generated containing the complete cytochrome P450 and the C-terminal appended simian V5 epitope and histidine (His_6) tag encoded by the vector. This tagging procedure allows detection of the expressed enzyme via immunoblot analysis of the isolated microsomal protein using commercially available antibodies, and does not compromise the activity of other recombinant taxoid hydroxylases [26]. Insert size was determined by restriction digests, followed by sequencing using the Gal1 (forward) and V5 C-term (reverse) primers (Invitrogen) to confirm orientation, and the verified clones were then transformed into the yeast host (S. cerevisiae strain WAT11 [31]) using the lithium acetate method [37].

In Situ Screening for Cytochrome P450 Function

Transformed yeast cells were grown to stationary phase in 2 ml of SGIA medium at 30°C with 250 rpm mixing. The cells were then harvested by centrifugation (2000g, 10 min) and the cell pellet was suspended in 3 ml YPLA galactose-containing induction medium. Approximately 9 h after induction, the cells were harvested again by centrifugation. For immunoblotting, the cells were resuspended in lysis buffer (100 mM Tris•HCl, pH 8.5, containing 1 mM DTT and 10% v/v glycerol), lysed by sonication (VirSonic, microtip probe, medium setting, 3 X 30 sec, VirTis Co., Gardiner, NY) or by use of a Bead Beater (Biospec Products, Bartlesville, OK), and the microsomes prepared [31]. Protein (50 μg) was then separated by SDS-PAGE (10% denaturing gel), transferred by wet transfer blotting to nitrocellulose and immobilized by UV-crosslinking. The appended C-terminal His_6-tag was then exploited by using mouse Penta-His-specific antibody (Qiagen, Valencia, CA) as primary antibody, and alkaline phosphatase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) as secondary antibody for detection. The Qiagen protocols were used throughout, with His-size markers as reference, and protein preparations from transformed cells harboring empty vector as negative controls.

Following confirmation of expression by immunoblot analysis, the activities of the recombinant cytochrome P450 enzymes were examined by in vivo feeding using a previously developed procedure [24], thus eliminating the uncertainties associated with microsome isolation and in vitro assay. For this purpose, the transformed and induced yeast cells that were harvested by centrifugation as above were resuspended in 3 ml of fresh YPLA medium to which 30 μM of the labeled test substrate was added, followed by overnight incubation at 30°C with mixing (250 rpm). The incubation mixture was then treated for 15 min in a sonication bath and extracted twice with 3 ml of hexane:ethyl acetate (4:1 v/v). The organic extract was then dried under N_2, the residue dissolved in 100 μl of acetonitrile, and an aliquot was separated by reversed-phase radio-HPLC [250 mm X 4.6 mm column of Alltech (Deerfield, IL) Econosil C_{18} (5 μm); flow rate of 1 ml/min; with radio-detection of the effluent (Flow-One-Beta Series A-1000, Radiomatic Corp., Meriden, CT)]. The following conditions were employed [solvent A: 97.99% H_2O with 2% CH_3CN and 0.01% H_3PO_4 (v/v); solvent B: 99.99% CH_3CN with 0.01% H_3PO_4 (v/v); gradient: 0-5 min at 100% (A), 5-15 min at 0-50% (B), 15-55 min at 50-100% (B), 55-65 min at 100% (B), 65-70 min at 0-100% (A), 70-75 min at 100% (A)]. The HPLC eluant was collected in 1 min fractions and the appropriate fractions containing the radiolabeled product were

combined, dried under a stream of N_2, and dissolved in the minimum volume of benzene for GC-MS analysis.

GC-MS analyses were performed on a Hewlett-Packard 6890 GC-MSD system using a ZB-5 capillary column [Phenomenex (Torrance, CA); 30 m length; 0.25 mm inner diameter; coated with a 0.25 μm film of phenyl (5%) polysiloxane]. Cool on-column injection was used, with He flow rate of 0.7 ml/min and a temperature program from 40°C to 320°C at 20°C/min. Spectra were recorded at 70 eV.

Cytochrome P450 cDNA Expression in Insect Cells

The functional expression of cytochrome P450 clones using Autographa californica baculovirus and Spodoptera frugiperda (Sf9) cells that coexpress the Taxus NADPH:cytochrome P450 reductase gene has been described [25]. For construction of the recombinant baculovirus harboring cytochrome P450 clone S1, the S1 ORF was amplified by PCR using Pfu DNA polymerase and gene-specific primers containing a BamHI site immediately upstream of the start codon and another containing a NotI site downstream of the stop codon. The gel purified amplicon was subcloned first into the pCR-Blunt vector (Invitrogen) and the insert then excised using the BamHI/NotI restriction sites and ligated into the similarly digested pFastBac1 vector (Life Technologies, Grand Island, NY). This pFastBac1 construct was then used to prepare recombinant Bacmid DNA by transforming Escherichia coli strain DH10Bac (Life Technologies) carrying the baculovirus genome. As a negative control for this expression system, recombinant baculovirus containing a β-glucuronidase gene, instead of the cytochrome P450 ORF, was used. Baculovirus construction and transfection of Sf9 cells were carried out according to the Life Technologies protocols, and culturing was performed as previously described [25].

Recombinant Enzyme Characterization and Other Assays

For microsome preparation, Sf9 cells were harvested three days after transfection, washed twice with 50 mM KH_2PO_4, pH 7.5, containing 9% (w/v) NaCl, twice with 50 mM Hepes, pH 7.5, containing 0.5 mM EDTA, 0.1 mM DTT and 10% (v/v) glycerol, and then lysed by gentle sonication as before in 50 ml of the Hepes buffer system. Cell debris was removed by centrifugation (10,000g, 20 min, 4°C), and the resulting supernatant was then centrifuged at 28,000g (20 min, 4°C) and then at 105,000g (120 min, 4°C) to provide the microsomal

membranes which were resuspended in the same Hepes buffer system without EDTA, or other buffer system as noted below. Protein content was determined by the Bradford method [38] using bovine serum albumin as standard.

CO-difference spectra [33] and substrate binding spectra [34] were recorded using a Perkin-Elmer Lambda 18 spectrophotometer [39]. The latter were recorded with up to 200 pmol of recombinant microsomal cytochrome P450 enzyme (determined by CO-difference spectral analysis) per cuvette in 100 mM sodium phosphate buffer at pH 7.5. In preparation for binding studies, the taxadiene isomers were each dissolved in DMSO and 1 µl additions to the sample were made to a final concentration of 1.5% (v/v). For data analysis, Spectrum for Windows (Perkin-Elmer Corp., Wellesey, MA) and Sigmaplot 7.0 (SPSS Inc., Chicago, IL) were employed and experiments were run in triplicate.

For catalytic assays, the isolated microsomes were resuspended in 50 mM Hepes, pH 7.5, containing 1 mM DTT and 5% (v/v) glycerol, and the 1 ml reactions (~600 µg protein, 50 µM substrate dissolved in DMSO, and the requisite cofactors) were run as described previously, with the identical protocols for product analysis [25]. DMSO was without influence on the reaction. For kinetic evaluation, following the establishment of linear reaction conditions in protein concentration and time, the response to substrate concentration was plotted by the Michaelis-Menten method (Sigmaplot 7.0) using the calibrated radio-HPLC protocol for product determination. Data from three independent experiments were pooled and the line of best fit taken ($R^2 > 0.99$).

Preparation of Taxus suspension cell microsomes and assay for the native taxadiene 5α-hydroxylase were carried out as previously described [14, 15] with the following modifications. Unelicited Taxus media hicksii cells were harvested 14 days after transfer, separated from the media, frozen in liquid N_2 and ground to a fine powder with a mortar and pestle, with extraction and microsome preparation as before [15]. The previously described radio-HPLC-based assay was employed [15] to separate the substrate from taxadien-5α-ol and polyols derived therefrom which were summed as "total product" for the purpose of rate determination.

The expression in E. coli of the taxadiene synthase allelic variants, and the preparation and assay of the recombinant taxadiene synthase isoforms, were conducted by established methods using capillary GC-MS conditions designed to separate taxadiene positional isomers [19].

The assay for microsomal taxa-4(5),11(12)-diene isomerase activity (and the reverse isomerization) was carried out under standard cytochrome P450 oxygenase conditions but in the absence of NADPH or O_2, or in the presence of inhibitory concentrations of CO, miconazole or clotrimazole (under conditions described previously for which the rate of 5α-hydroxylation is negligible [14]). A number of additional, potential cofactors were also tested, including FAD, $FADH_2$, FMN, $FMNH_2$, NAD^+, NADH and $NADP^+$ (all at 2.5 mM), as well as $MgCl_2$ (at 5.0 mM). The possibility of pH-dependent isomerization was tested by incubating each isomer (100 μM) in phosphate buffer (pH 4 to 10) for 12 h at 31°C, with separation of isomers as before [19].

Acknowledgments

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Accession Number

The complete DNA sequence of taxadiene 125a-hydroxylase has been deposited in the GenBank database with accession number AY289209.

Figure Legends

Figure 1. Outline of the early steps of taxol biosynthesis. Taxol (**1**) formation involves the cyclization of geranylgeranyl diphosphate (**2**) to taxa-4(5),11(12)-diene (**3**) and cytochrome P450-mediated hydroxylation to taxa-4(20),11(12)-dien-5α-ol (**4**).

Figure 2. Deduced amino acid sequence alignment of taxoid hydroxylases. The sequences of taxoid 10β-hydroxylase (T10H), taxoid 13α-hydroxylase (T13H) and the clone S1 taxadiene 5α-hydroxylase (T5H) are compared. Black boxes indicate identical residues for the three sequences; grey boxes indicate identical residues for two of the three.

Figure 3. Substrate binding spectra of taxadiene isomers. Microsomes from S. frugiperda cells enriched with 200 pmol recombinant taxadiene 5α-hydroxylase (clone S1) were employed. Taxa-4(20),11(12)-diene was assayed over a concentration range from 0.1 to 10 μM, and a binding constant (K_s) of 4 ± 1 μM was determined (**A**). Taxa-4(5),11(12)-diene was assayed over a concentration range of 0.1 to 20 μM and a binding constant (K_s) of 6.5 ± 1.5 μM was determined (**B**).

Figure 4. Kinetic evaluation of taxadiene isomers. Taxa-4(20),11(12)-diene (○) and taxa-4(5),11(12)-diene (●) were evaluated with microsomes from S. frugiperda cells enriched with 50 pmol recombinant taxadiene 5α-hydroxylase (**A**), and with microsomes from T. media suspension cells containing about 50 pmol of total native cytochrome P450 (**B**). Substrate concentration range was varied from 1 to 500 μM in all cases. Taxa-4(20),11(12)-diene yielded

an average $\underline{K}_m$ value of 21.5 μM with V_{rel} of 135, and taxa-4(5),11,12-diene yielded an average $\underline{K}_m$ value of 36 μM with V_{rel} of 100.

Figure 5. Proposed mechanism for cytochrome P450 taxadiene 5α-hydroxylase. This cytochrome P450-mediated conversion of taxa-4(5),11(12)-diene (**3**) and taxa-4(20),11(12)-diene (**5**) to taxa-4(20),11(12)-dien-5α-ol (**4**) is thought to involve hydrogen abstraction from C20 (in **3**) or C5 (in **5**) to provide a common allylic radical intermediate, followed by oxygen insertion at the 5α-face to yield taxadien-5α-ol (**4**). Isomerization of **3** to **5** was not observed, nor does the route via epoxide **6** with rearrangement seem likely.

Figure 6. Nucleic acid sequence of Taxediene 5α-hydroxylase (Clone S1) cDNA.

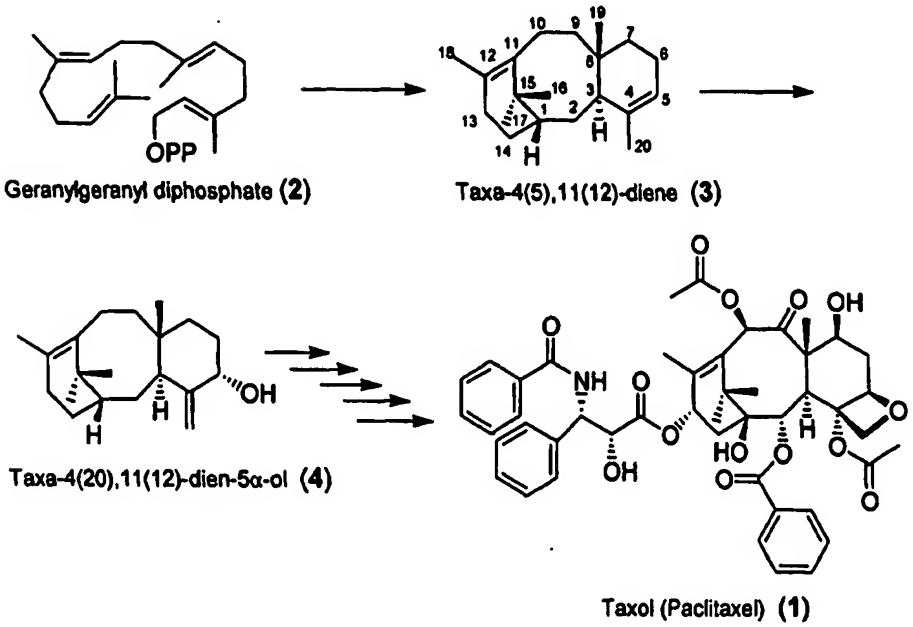
OPP
Geranylgeranyl diphosphate (2)
Taxa-4(5),11(12)-diene (3)
OH
Taxa-4(20),11(12)-dien-5α-ol (4)
Taxol (Paclitaxel) (1)

FIGURE 1

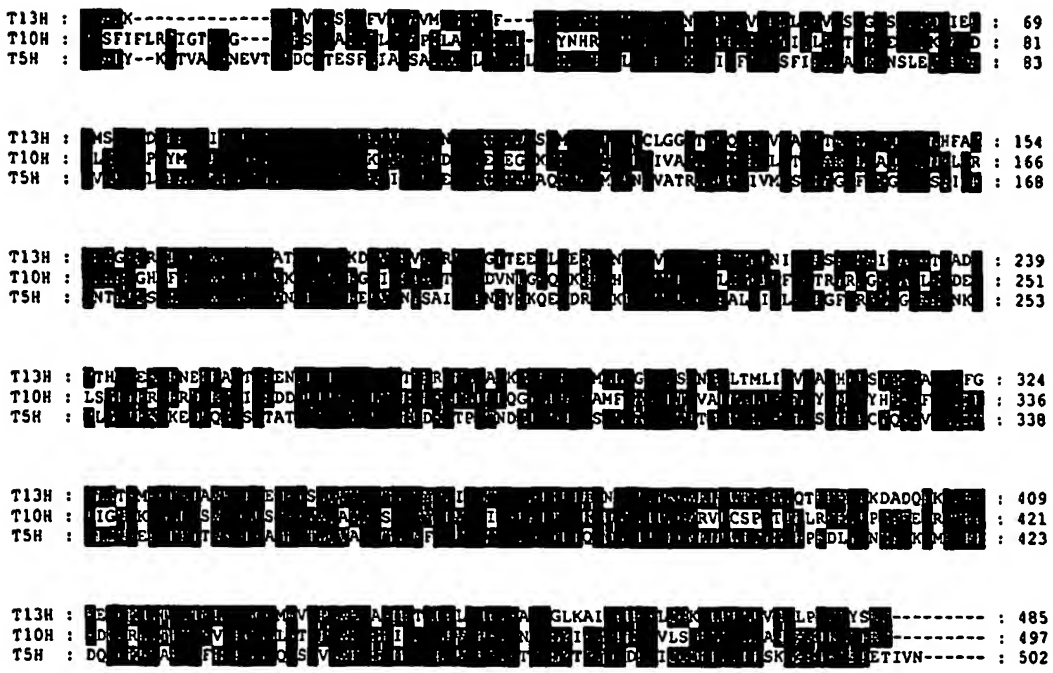

FIGURE 2

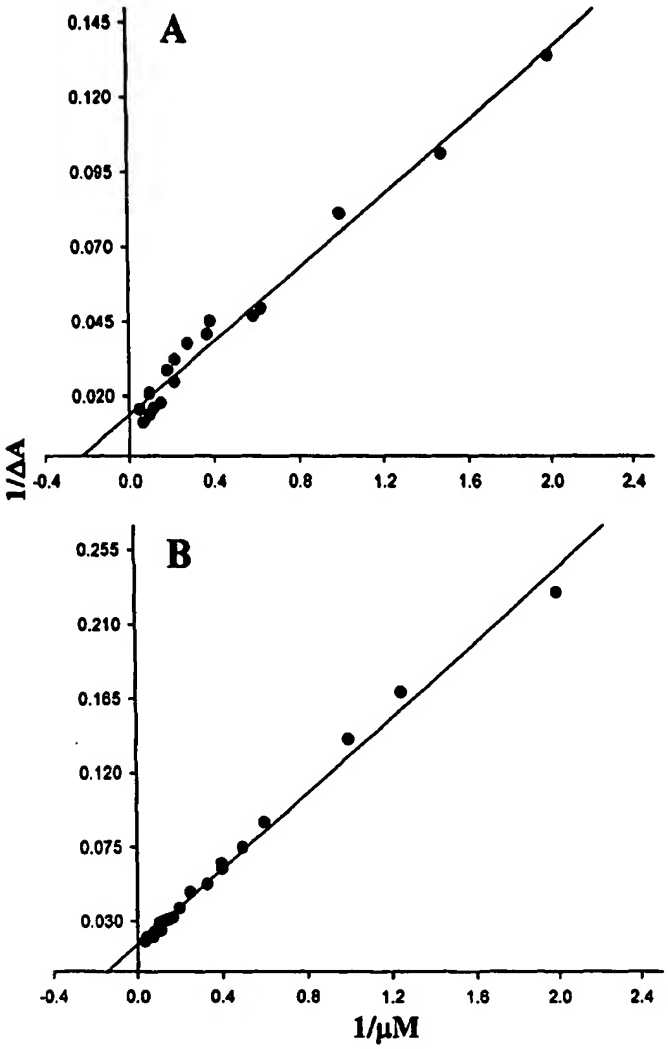
A
B
0.145
0.120
0.095
0.070
0.045
0.020
0.255
0.210
0.165
0.120
0.075
0.030
-0.4
0.0
0.4
0.8
1.2
1.6
2.0
2.4
1/ΔA
1/μM

FIGURE 3

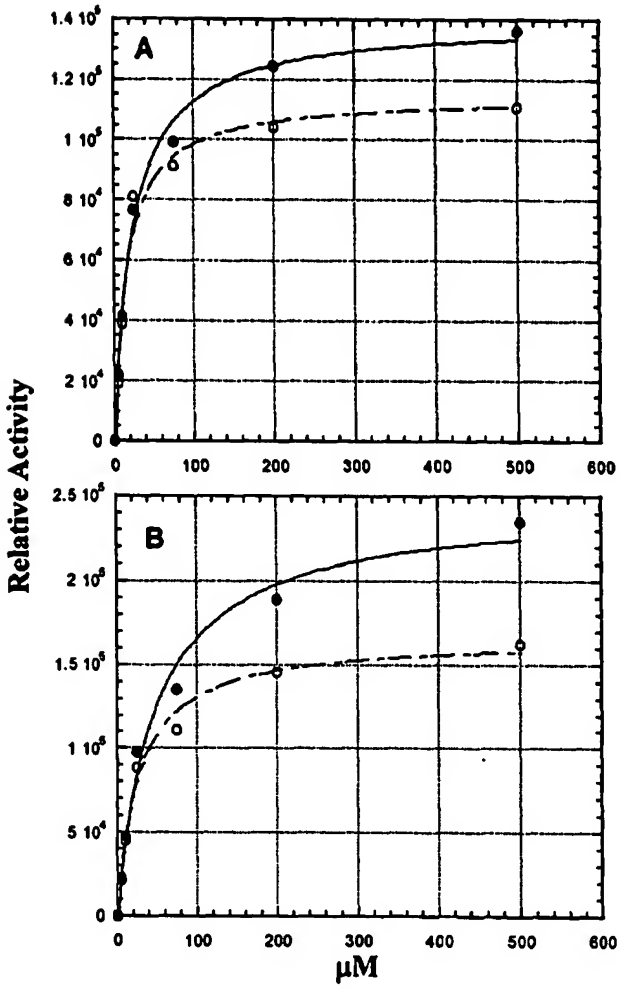

FIGURE 4

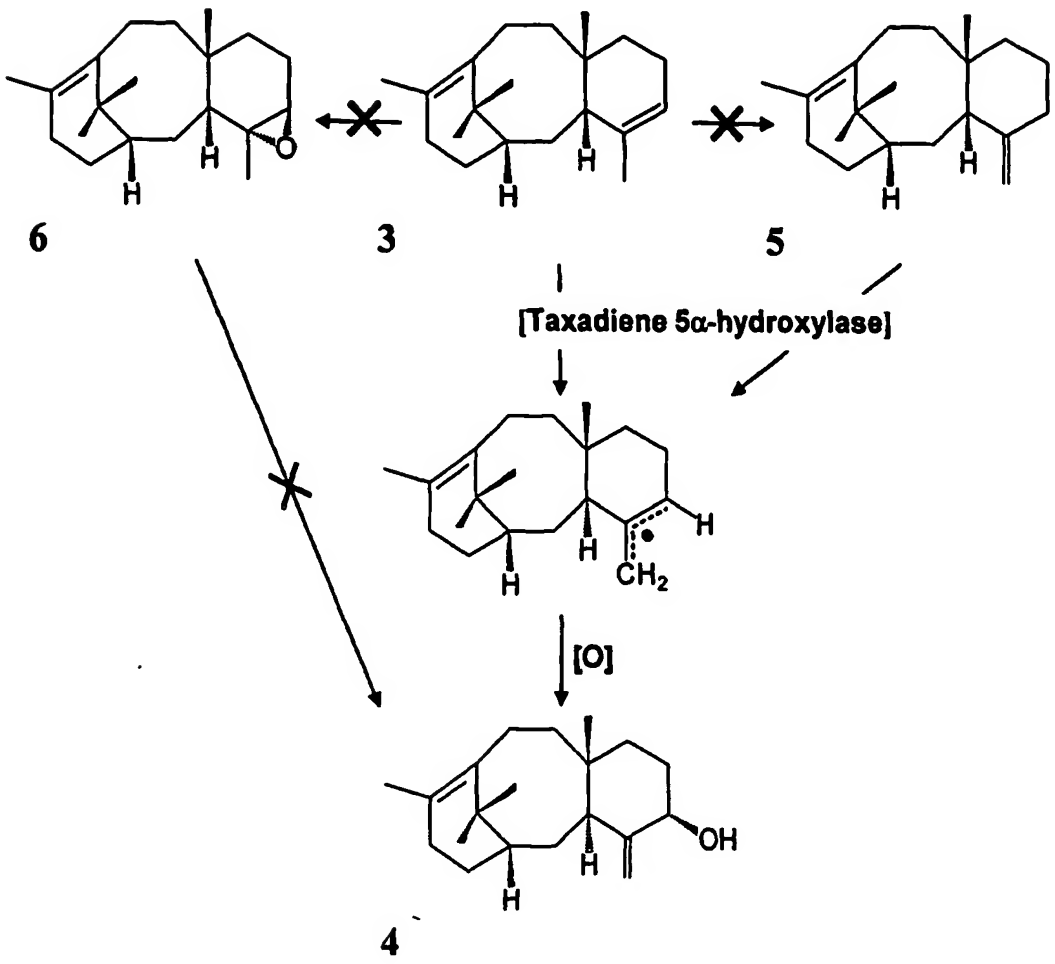

FIGURE 5

Taxane-5-hydroxylase cDNA sequence

CGGCACCAGGTTTTCTGCTCCTGCTTTTCTTCTTCCAAAATGGACGCCCTGTATAAGAGCACAGTTGCAAAATTTAAT
GAGGTCACACAGCTGGACTGTTCCACTGAATCTTTTTCCATTGCCCTCTCAGCTATTGCTGGTATTCTTCTGCTTCTC
CTGCTCTTCCGTTCTAAACGCCACTCCTCCCTTAAACTTCCTCCTGGGAAATTAGGCATCCCTTTCATTGGCGAGTCG
TTTATCTTCCTGAGGGCTCTTCGATCGAACTCGCTGGAGCAATTTTTTGACGAGAGAGTGAAGAAATTCGGCCTCGTG
TTCAAGACCTCCTTGATTGGGCATCCCACAGTAGTACTCTGCGGCCCTGCGGGAAACCGGCTTATTCTGTCCAACGAG
GAGAAGCTGGTGCAGATGTCGTGGCCCGCTCAATTTATGAAGCTCATGGGGGAGAATTCCGTTGCCACCAGGAGGGGT
GAAGACCATATAGTTATGCGCTCTGCTCTTGCAGGTTTTTTCGGCCCTGGTGCGCTGCAGAGTTACATTGGTAAAATG
AATACAGAGATCCAGAGTCATATCAACGAAAAATGGAAGGGAAAAGATGAGGTGAATGTACTTCCTTTGGTAAGAGAG
CTCGTCTTCAACATTTCGGCCATCTTGTTTTTCAACATATATGATAAGCAGGAACAGGATCGTCTGCATAAGCTTTTG
GAAACTATTCTGGTCGGAAGTTTTGCTCTTCCGATTGACTTGCCCGGATTTGGTTTCCATAGAGCACTCCAGGGACGG
GCCAAGCTCAACAAAATTATGCTGTCTTTAATTAAAAAGAGAAAAGAAGATCTGCAGTCTGGATCGGCAACAGCCACG
CAGGATCTGCTCTCTGTTTTGCTCACTTtCAGAGATGaCAAAGGGACTCCACTCACCAATGATGAGATACTCGACAAC
TTTTCTTCTCTGCTCCATGCCTCCTATGACACCACCACTTCGCCAATGGCTTTGATTTTCAAGCTCTTGTCTTCCAAT
CCAGAATGCTATCAAAAAGTAGTTCAAGAGCAATTGGAGATACTTTCCAACAAAGAGGAGGGCGAAGAAATCACATGG
AAGGATCTCAAAGCCATGAAATACACATGGCAAGTAGCTCAGGAAACGCTGCGGATGTTTCCTCCAGTTTTCGGAACA
TTTCGCAAGGCCATCACTGACATTCAGTATGATGGTTACACAATTCCAAAAGGGTGGAAGCTGTTGTGGACAACTTAC
AGTACACATCCCAAGGACTTGTATTTCAATGAACCAGAGAAATTCATGCCTTCAAGATTCGATCAGGAAGGAAAGCAT
GTAGCTCCTTACACATTTTTGCCCTTCGGTGGAGGCCAACGGTCATGTGTGGGATGGGAATTTTCAAAGATGGAGATA
TTACTATTCGTTCATCATTTTGTCAAAACTTTTAGCAGCTACACCCCAGTTGATCCCGACGAAAAAATATCAGGGGAT
CCACTCCCTCCTCTTCCTTCCAAGGGATTTTCCATTAAACTGTTTcCCGAGACCATAGTCAATTGAAGGAGAAAACCA
CAGTGCAGAACTGCTATTCTTGAATCCTCGCTCAAGAATAATACAAACATGCATCACCAACAATGTTTATGCACTCAA
TGCAAATTAACAGTGTGTCAGCATTGACAGTCAAAAAAAAAAAAAAAAAA

FIGURE 6